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RESEARCH ARTICLE

IDENTIFICATION OF SPECIFIC SEQUENCE OF *Beauveria bassiana* FOR THE SELECTIVE STRAIN WITH THE RAPD MARKER SELECTION

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ABSTRACT

In this study we developed strain specific Random Amplified Polymorphic DNA molecular markers based on Polymerase Chain Reaction amplification of specific sequence in combination with dilution plating on semi-selective medium to detect and estimate density of propagules of a commercial strains of B. bassiana. Using Random Amplified Polymorphic DNA (RAPD) analysis, the fifteen primers of four Operon series (OPB, OPC, OPE and OPF) were selected for 16 accessions of B. bassiana. All Three Operon series primers (OPB-2, OPB-4 and OPC-4) were highly sensitive and yielded maximum amplification products with all Beauveria bassiana accessions. Analysis of Sixteen accessions of Beauveria bassiana revealed 90.97% of polymorphism. Besides molecular characterization of Beauveria isolates, RAPD markers proved to be very useful in selecting and identifying the specific sequences of B. bassiana for the selective strains which can be developed in future as a more effective bio-control agents.

INTRODUCTION

Study conducted on the efficacy and persistence of a microbial control agent require identification of specific sequences of selective strain of *Beauveria bassiana* with highly polymorphic molecular markers. These techniques are critical for the entomopathogenic fungus *Beauveria bassiana* because the fungus has a wide insect host range and is common in nature (McCoy *et al*., 1988). There are also various *B. bassiana*–based mycoinsecticides currently registered or under commercial development worldwide for agricultural pests (Hajek *et al*., 2001). Molecular markers have been utilized to assess genetic variation among isolates of B. bassiana and other entomopathogenic fungi, thereby providing means to identify strains of interest, determine origin of isolates, or study population structure. One technique that has been used to differentiate strains of *B. bassiana* is polymerase chain reaction (PCR) based random amplified polymorphic DNA (RAPD) (Bidochka *et al*., 1994; Castrillo *et al*., 1999; Maurer *et al*., 1997). This technique utilizes short primers of arbitrary sequence that anneal to multiple target sequences producing diagnostic patterns (Wiliams *et al*., 1990). Because RAPD analysis does not require prior knowledge of target site sequences, it can be easily adapted to study various entomogenous fungi, even those with poorly studied genomes.

RAPD analysis has also been utilized to generate unique PCR products or amplicons in filamentous fungal species or strains of interest and identification of specific sequence of *Beauveria bassiana* for the selective strains (Abbasi *et al*., 1999; Lecomte *et al*., 2000; Li *et al*., 1999; Schilling *et al*., 1996). RAPD primers are designed based on known DNA sequences of the organism in the study. This allows for the development of sensitive and diagnostic assays to amplify specific fungal DNA in selective strains containing mixed DNA because primers anneal specifically to fungal sequences. This is in contrast to RAPD analysis that requires the establishment of single spore isolates for strain identification. In this study we utilized RAPD–PCR technique to screen for markers that would differentiate the *B. bassiana* strains of the fungus. These RAPD markers develope a sensitive diagnostic assay for the selective detection of *B. bassiana* strains or isolates.

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MATERIALS AND METHODS

Selection of Fungal isolates

Sixteen accessions of *Beauveria bassiana* were taken from Scientific And Applied Research Center, laboratory Meerut for the study. Seven were Exotic Collections (EC) from (USA, Rashia, Philippines, Taiwan and Philippines) and Nine were Indigenous Collections (IC) from different parts of the country. All the accessions of *B. bassiana* were kept at 4°C as cultures on SDA or Potato Dextrose Agar. The indigenous accessions were randomly selected to include the maximum variability by virtue of their place of collections, representing four states of India, (Uttar Pradesh, Gujarat, Andhra Pradesh and Jharkhand). These 16 accessions of *Beauveria bassiana* were grown in the Randomized Block Design (RBD) as per the following layout plan during 2010-11and 2011-12. The experimental material was grown in the RBD.

Plant DNA Isolation

Extraction of total genomic DNA was carried out as described by (Murray et al., 1980) with minor modifications. Leaf tissue of 2gm was placed in autoclaved mortar, by adding liquid nitrogen, crushed vigorously with a pestle to a fine powder, care being taken to prevent thawing of the material. The powder was transferred to an autoclaved 50 ml polypropylene centrifuge tube containing 15ml of pre-warmed

(65°C) DNA extraction buffer to disrupt the cell membranes. The tubes were incubated at 65°C in a water bath for one hour after mixing vigorously. The contents were mixed gently during the incubation period by inverting the tube once every 15 minute. Tubes were cooled to room temperature and equal volumes of chloroform: isoamyl alcohol solution (24:1) were added and mixed by gentle inversion for 5 min. The contents were then centrifuge for 10 min at 10,000 rpm at temperature 10-25°C. The aqueous phase was transferred to fresh centrifuge tubes with wide bore tips to avoid DNA shearing and 0.6 ml volume of chilled isopropanol was added. The content was mixed by gentle inversion and incubated at -20ºC for 30 minutes for DNA precipitation. The precipitated DNA obtained was pooled out by centrifuge at 12,000 rpm for 15 minutes. Discard the aqueous phase and DNA pellet was washed twice with 70% ethanol and centrifuged at 12000 rpm for 10 minutes. Each time, and keep the pellet to air dry. After drying dissolve in 100 µl of TE buffer (pH-8.0) and kept in a refrigerator at 4°C. DNA concentration was determined by use of a spectrophotometer or by running aliquots of DNA extracts against a molecular mass ladder (Life Technologies).

Primer Design

A preliminary screening was conducted using six B. bassiana strains, against 10-nucleotide random primers obtained from Operon Technologies. A total of 56 RAPD primers were screened, from which four primers producing robust, reproducible, and unique amplicons of less than 1kb in B. bassiana strains were selected. The fifteen primers of four Operon series (OPB, OPC, OPE and OPF) were selected for 16 accessions of B. bassiana.

RAPD Analysis and PCR Amplifications

Polymerase Chain Reaction (PCR), a molecular biological technique for creating multiple copies (amplifying) of DNA without using a living organism, was used for amplification of gene to confirm the presence of this gene in isolated fungus. The procedure described by Williams *et al.*, 1990 with minor modifications was used for carrying out the PCR reactions for RAPD analysis. PCR reaction were set up in 25µl volume containing 25ng of *B. bassiana* genomic DNA, 5pmole (13ng) of decamer primers, 0.1mM dNTPs, 10x PCR buffer (10mM Tris, pH-8.0,50 mM KCL and 50mM Ammonium Sulphate), 1 mM $MgCl_2$, and 0.5 unit of Taq DNA polymerase. The volume was made up to 25 µl by autoclaved double distilled water.

The cocktail was subjected to PCR amplification in a Thermal cycler (biometra, USA). The PCR cycling condition involves initial denaturation at 94º for 5 minutes, followed by 40 cycles of:

- Denaturation at 94º C for 1 minute.
- Primer annealing at 35°C for 1 minute.
- Extension at 72°C for 2 minutes.

Final extension step at 72°C for 7 minutes followed by storage at 4°C before electrophoresed.

Amplified DNA fragments were resolved by submerged horizontal slab gel electrophoresis in 1.4% (w/v) agarose gel. The gel was prepared by dissolving agarose in 1xTAE buffer and heated for dissolution. Gel was cooled to 60-65oC and visualized by staining with ethidium bromide (Sigma, USA) was added at the rate of 4µl/100ml.The gel was poured into the cassettes with combs and allowed to polymerize at room temperature. The gel along with tray and combs were shifted to the electrophoretic tank with (1x) TAE buffer and combs were removed carefully. PCR samples were prepared by mixing 5ul of 6x loading dye, vortex for proper mixing and loaded in the preformed wells. Electrophoresis was carried out in 1x TAE buffer at 80 volts for 4 hrs till bromophenol blue/loading dye migrated to other end of the gel. Gel photos were taken using gel documentation system (alphaimagner TM image acquisition with CCD camera, san leandeo California), amplicons were scored as discrete variables, using 1 to indicate presence, 0 for absence. Each RAPD assay was done three times to ensure reproducibility.

RESULTS AND DISCUSSION

For RAPD analysis four Operon series (OPB, OPC, OPE and OPF) were tested out of which most reproducible primers were used for the fingerprinting. In the present study Fifteen RAPD primers were used for the fingerprinting, four each from OPB, OPC and OPE series and three from OPF series (Table 1).

Table 1. Primer sequence and amplification pattern produced by 15 random primers of sixteen *Beauveria bassiana* accessions.

Primer	Sequences	Band	Monomorphic	Polymorphic
OPB-01	GTTTCGCTCC	10	0	10
OPB-02	TGATCCCTGG	12	2	10
OPB-03	CATCCCCCTG	10	0	10
OPB-04	GGACTGGAGT	14	0	14
OPC-01	TTCGAGCCAG	10	0	10
OPC-02	GTGAGGCGTC	8	6	2
OPC-03	GGGGGTCTTT	10	0	10
OPC-04	CCGCATCTAC	14	0	14
OPE-01	CCCAAGGTCC	9	0	9
OPE-02	GGTGCGGGAA	8	0	8
OPE-03	CCAGATGCAC	12	4	8
OPE-04	GTGACATGCC	8	0	8
OPF-01	ACGGATCCTG	9	0	9
OPF-02	GAGGATCCCT	6	1	5
OPF-03	CCTGATCACC	4	l0	4
	TOTAL	144	13	131

All the primers used in the present study were polymorphic, number of bands produced by each primer varied from 14, only in case of OPC2 and OPF3 it has produced 2 and 4 bands respectively. All the bands produced in the present study were scored for the analysis. Fifteen primers were used in the study of RAPD markers analysis to standardization of suitable specific primers amplifying the genetic materials of *Beauveria bassiana* accessions. All primers but three (OPB-2, OPB-4 and OPC-4) yielded maximum amplification products with all *Beauveria bassiana* accessions. The primers amplified DNA products from each *Beauveria bassiana* accession generating reproducible band patterns. The remaining primers gave patterns that were identical or had differences too small to provide information on the genetic diversity. Analysis of Sixteen accessions of *Beauveria bassiana* revealed 90.97% of polymorphism. The total 144 bands were scored for the 15 RAPD primers out of which 13 bands were monomorphic. Using NTSYS software Jaccard's similarity coefficient were calculated for each accession. Then based on Jaccard's similarity coefficient Dendrogram was constructed for each genotype of *Beauveria bassiana.* The PCR amplified band patterns of Sixteen accessions of *Beauveria bassiana* were shown in Fig 1.

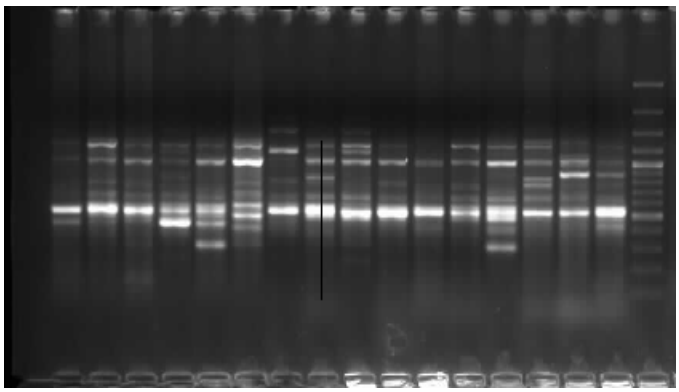

Fig. 1. RAPD primer used for PCR amplification of 16 accessions of *B.bassiana* namely (1) EC388892, (2) IC75730, (3) IC326735, (4) IC388895, (5) IC201233, (6) EC344638, (7) EC 388896, (8) IC326732, (9) EC388889, (10) IC344681, (11) IC369247, (12) IC434653, (13) EC112548, (14) IC387837, (15) EC3888788, (16) 388990

Genetic similarity measured through analysis of RAPD data of Sixteen accession of belonging to two *Beauveria bassiana* revealed varying degree of genetic relatedness among accessions belonging to different species, highest similarity (.71) was measured between accession IC-369247 and IC-201233 and least similarity (.34) was measured in IC-387837 and EC-388896 given in Table-2.

Table 2. Similarity indices of RAPD markers of *Beauveria bassiana* species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.00															
2	0.63	1.00														
3	0.54	0.51	1.00													
4	0.56	0.62	0.40	1.00												
5	0.53	0.59	0.49	0.58	1.00											
6	0.50	0.54	0.43	0.59	0.61	1.00										
7	0.47	0.48	0.39	0.58	0.53	0.60	1.00									
8	0.50	0.58	0.45	0.62	0.57	0.57	0.66	1.00								
9	0.49	0.57	0.41	0.60	0.58	0.62	0.64	0.67	1.00							
10	0.46	0.50	0.41	0.51	0.55	0.65	0.54	0.56	0.63	1.00						
11	0.44	0.51	0.41	0.53	0.71	0.69	0.55	0.53	0.59	0.69	1.00					
12	0.47	0.52	0.38	0.53	0.57	0.63	0.45	0.53	0.59	0.55	0.62	1.00				
13	0.44	0.50	0.42	0.51	0.56	0.56	0.58	0.58	0.70	0.54	0.56	0.53	1.00			
14	0.45	0.52	0.44	0.48	0.50	0.52	0.34	0.54	0.60	0.51	0.55	0.70	0.58	1.00		
15	0.52	0.54	0.51	0.55	0.63	0.53	0.49	0.61	0.55	0.52	0.60	0.59	0.50	0.55	1.00	
16	0.53	0.59	0.46	0.63	0.55	0.68	0.54	0.54	0.65	0.60	0.53	0.64	0.54	0.57	0.60	1.00

And the phylogenetic tree comprising a total of Sixteen accessions of *Beauveria bassiana* RAPD markers was constructed as shown in Figure -2. The correlation coefficient calculated between RAPD when using the similarity 90.97% when using the dendrogram. The number of genetic loci detected with RAPD markers are much higher than detected with morphological and chemical / biochemical markers (Kongkiatngam *et al*., 1995). In the dendrogram, the Sixteen accessions were divided into two major groups. The second major group sub divided into two sub groups. The first sub group includes IC-388895 and IC-75730 showed similarity with EC-388892. The second sub group was further sub divided into three sub groups, where the first sub group includes EC-388990 and EC-388788 the second sub group include IC-344681 with two genetic similar accessions IC- 364247 and IC- 201233, IC-344638 accession was also similar with these accessions. IC- 434653 and IC-387831 was also seems to be similar with accessions of second sub group. The third sub group includes IC-326735 and IC-326732 with EC-388889 and EC- 112548.The first major group include EC-388896 accession only. The similarity and differences of accessions among these groups is expected because of their genetic resemblance or genetic divergence. A result of this study suggests that the molecular diagnoses of cultivars of *Beauveria bassiana* differ very little among themselves, observations suggest that the genetic base utilized in their breeding programs and these observations consider for utilization in plant improvement program.

Conclusion

Beauveria bassiana species are valued as spice plants in India. Driven by commercial incentives, the wild population of this plant has been threatened with depletion in recent years due to excessive harvesting. The present study was preliminary attempt to develop RAPD primers to distinguish the sixteen *Beauveria bassiana* genotypes shown that more difficult screening of primers has to be done before RAPD markers can be developed. This study showed a significant morphological variation and a large genetic diversity within and among cultivars. Lately, this technique has been used to study the genetic relations between the different species of coffee and to determine the relationship between hybrids.

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